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*Original Article*

# Long non-coding RNA Xist promotes progression of non-small-cell lung cancer (NSCLC) by modulating miR-103a and MAP3K3 pathway

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**Abstract:** Background: Non-small-cell lung carcinoma (NSCLC) is the most common type of lung cancer with poor prognosis despite advent of newer treatment modalities. The role of long non-coding RNA (lncRNA)-Xist in cancer including NSCLC has already been established. Hence, understanding the underlying mechanism of action of lncRNA-Xist in NSCLC might provide basis for novel drug targets. Objective: In this study, we explored the underlying molecular mechanism through which lncRNA-Xist promotes NSCLC progression. Materials and methods: In the human lung cancer cell line A549, cell proliferation and percentage of apoptotic cells were measured by MTT assay and flow cytometry. Western blot analysis was done to explore the expression level of MAP3K3. Real-time PCR was used for RNA analysis. LncRNA-Xist silencing was done by infecting the A549 cells by lentivirus encoding shRNA which targeted the lncRNA-Xist. Results: The results showed a significant increase in the expression of lncRNA-Xist while a decrease in the miRNA103a expression ($P<0.05$). Silencing of lncRNA-Xist led to suppression of cell proliferation and induction of apoptosis ($P<0.05$). Also, lncRNA-Xist knockdown led to significant increase in expression of miRNA-103a, which in turn caused suppression of A549 cell proliferation ($P<0.05$). Finally, lncRNA-Xist knockdown and miR-103a overexpression independently down-regulated MAP3K3 pathway. Conclusion: lncRNA-Xist promotes progression of NSCLC by down-regulating the expression of miRNA-103a which can suppress cell proliferation and induce apoptosis and by up-regulating MAP3K3 pathway.

**Keywords:** Non-small-cell lung carcinoma, long non-coding RNA-Xist, MiRNA-103-a, MAP3K3 pathway

## Introduction

Lung cancer is one of the most common cause MAP3K3 pathway of cancer-related deaths in men whereas in women it stands second. About 14% of all new cancers are lung cancers. Lung cancer is responsible for about one out of 4 cancer deaths; cancer deaths due to lung cancer exceeds those due to colon, breast, and prostate cancer combined [1]. Non-small-cell lung carcinoma (NSCLC) is the most frequent type of lung carcinoma accounting for about 85% of lung cancer varieties. Despite recent progress in treatment modalities for NSCLC, the 5-year survival rate is still very low (less than 18%) [2].

Noncoding RNAs (ncRNAs) belong to a particular variety of RNA transcripts which do not translate into protein. Other less commonly used terms are non-protein-coding RNA (npcRNA), non-messenger RNA (nmRNA), functional RNA (fRNA), etc. There are mainly two classes of ncRNA namely housekeeper ncRNAs (like rRNA, tRNA, snRNA, etc), and regulated ncRNAs [3]. The regulated ncRNAs are further categorized by the length of the nucleotide (nt); short ncRNAs, with <200 nt, and long ncRNAs (lncRNAs) with >200 nt.

Iyer MK et al, in his recent study published that there are approximately 60,000 ncRNAs, of which more than 68% are lncRNAs [4]. lncRNAs are associated with the process of tumorigenesis which affects tumor initiation, progression, and metastasis through modulation of oncogenic and tumor suppressing pathways [2, 3, 5-7].

The exact functions of most of the lncRNAs are yet to be elucidated, however, currently sev-

eral lncRNAs have been implicated in various types of cancers [5]. Previous studies have already established the roles of ANRIL [8], AK001796 [9], BCYRN1 [10], and HNF1A-AS1 [11] in the induction of cellular migration and metastasis in NSCLC. Similarly, expression of HIF1A-AS1, another type of lncRNA, is upregulated in a number of renal cancer cases hinting at the possibility of its involvement in tumorigenesis [12]. The lncRNA-Xist (X-inactive specific transcript, a product of the XIST gene) is the most important regulator of X chromosome in mammals [12]. It is already established that the expression of XIST is increased by many folds in certain cancers like glioblastoma [13], breast [14], and ovarian cancer [15]. Furthermore, these findings are supported by the *in vivo* study in which tumor growth was suppressed in XIST knockdown nude mice and showed an improved survival of the mice [16]. Despite these findings, the underlying mechanism through which lncRNA-Xist promotes NSCLC are yet to be established unequivocally [5].

MicroRNAs (miRNAs) are single stranded non coding endogenous RNA molecules with about 19-25 nucleotides. miRNAs bind to complementary recognition sequences of mRNA and can regulate the expression of mRNA targets. It has been established that each miRNA can regulate the expression of more than one RNA and vice versa [16-18] and has been estimated to target more than 30% of the protein-coding genes. Several biological processes like cell differentiation, proliferation, apoptosis, and metabolism are regulated by miRNAs [19]. Of the different types of miRNA, miR-103a has been associated with different types of cancers such as pancreatic, bladder, nasopharyngeal, colorectal, lung cancers, etc [20].

Mitogen-activated protein kinase (MAPK) pathways are implicated in various processes like cellular proliferation, differentiation, migration, and apoptosis [21]. MAPK pathways consist of three-tiered kinase modules; MAPK is activated by a mitogen-activated protein kinase kinase (MAPKK), which is in turn activated by mitogen-activated protein kinase kinase kinase (MAP3K3, MAPKKK), located on the long arm of chromosome 17 (17 q) (23). Abnormalities in the MAPK signaling pathways resulted in both development and progression of cancer [20-22].

Hence, in this study, we explored the underlying molecular mechanism through which lncRNA-Xist promotes NSCLC progression.

## Materials and methods

### *Cell culture*

Human lung cancer cell line A549 was cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA). The media was supplemented with 10% fetal bovine serum (FBS; Gibco). All A549 cells were cultured at 37°C in a humidified incubator under 5% $CO_2$ (24).

### *Cell proliferation assay*

Cell metabolic activity was assessed using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium proliferation assay [23]. On each day of the MTT assay, 100 µL of cells was taken from each of the culture media and placed, in triplicate, in a 96-well plate Fifty micrograms of MTT was added to each well and this mix was incubated for 4 hours at 37°C. At the end of the incubation period, 100 µL of 0.04 N HCl in 2-propanol was mixed thoroughly into each well. Plates were read with a Molecular Devices microplate reader (Sunnyvale, CA) at a wavelength of 570 nm, with a background reading at 650 nm subtracted. Triplicate readings for each sample were averaged.

### *MicroRNA transfection*

miRNA precursors were purchased from Ambion (Austin, TX). Synthetic miR-103a mimic and scrambled negative control RNA were purchased from Genepharma (Shanghai, China). Cells were seeded in 6-well plates and were transfected with Lipofectamine 2000 (Invitrogen) with approximately 70% cell confluency [24]. In each well, equal amount (100 pmol) of either miR-103a mimic, or the scrambled negative control RNAs was used. The efficiency of down-regulation of overexpression of miR-103a was evaluated by reverse transcription polymerase chain reaction (RT-PCR).

### *Estimation of lncRNA-Xist expression: infection with lentivirus encoding short hairpin RNA*

Lentivirus (GV118) prepared by GenePharma (Shanghai, China) was used to encode short hairpin RNA (shRNA) targeting lncRNA-Xist [7]. The negative control of shRNA was also pre-

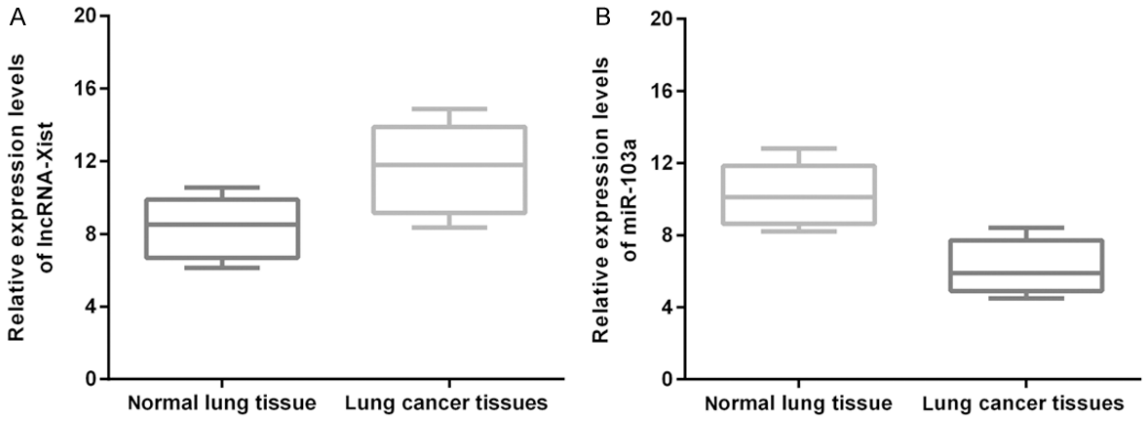


**Figure 1.** Expression of lncRNA-Xist in human lung cancer tissue and cells. A: Comparison of expression of lncRNA-Xist between normal lung tissue and in human lung cancer tissues (A549 cells). B: Comparison of expression of miR-103a between normal lung tissue and in human lung cancer tissues (A549 cells).

pared by GenePharma (Shanghai, China). A549 cells were kept in 6-well plates at a density of $1\times10^4$ cells/well and incubated for 12 h at 37°C. The culture medium was then replaced with fresh DMEM, and the cells were immediately infected with shRNA lentiviruses at a multiplicity of infection (MOI) of one (2 μL). After 24 h of growth at 37°C, the medium was replaced with fresh medium; the cells were cultured for 96 h at 37°C. The knockdown efficiency at the mRNA level was assessed using quantitative RT-PCR.

*Apoptosis assay by Annexin V-FITC/PI-staining*

Cells ($3\times10^6$) were stained using the FITC Annexin V/Dead Cell Apoptosis Kit (V13242, Invitrogen) according to the manufacturer's instructions [25]. Stained cells were diluted in Annexin V-binding buffer (Invitrogen). Then the suspended cells were used to perform flow cytometry. Annexin V-FITC/PI-stained cells were analyzed using a BD FACS Calibur flow cytometer (BD Biosciences, Heidelberg, Germany). In total 10,000 cells were analyzed per measurement. Data was analyzed using FlowJo 10.0.7 software (TreestarInc, Ashland, US).

*Western blot analysis*

The cells were washed 2 times with PBS and then lysed with 1×SDS loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) as the whole-cell sample. The protein samples were subjected to SDS-PAGE gel electrophoresis [26]. Immunoblotting were carried out with primary antibodies (anti-MAP3K3, anti-beta-actin, Cell Signaling). The proteins were detected by enhanced chemiluminescence (ECL-plus, Amersham Pharmacia Biotech).

*RNA extraction and quantitative real-time PCR analysis*

Total RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, NY). RNA (500 ng) was polyadenylated and reverse transcribed to cDNA using an NCode miRNA First-Strand cDNA synthesis kit (Invitrogen) [27]. cDNA was used as the template for real-time PCR FastStart Universal SYBR green Master (Roche) with the universal reverse primer provided in the kit. Real-time PCR was performed on Applied Biosystems real-time detection system (Applied Biosystems), and the thermocycling parameters were 95°C for 3 min and 40 cycles of 95°C for 15 s followed by 60°C for 30 s. Each sample was run in triplicate and was normalized to U6 snRNA levels (U6 primers 5'-CTTCGGCAGCACATATACT-3' [forward] and 5'-AAAATATGGAACGCTTCACG-3' [reverse]). Melting curve analysis was performed to confirm the specificity of the PCR products. The replicates were then averaged, and the fold induction was determined by a $\Delta\Delta C_T$-based fold change calculation.

*Statistical analysis*

Statistical analysis was performed using SPSS 19.0 (IBM Corporation, Armonk, NY, USA). The

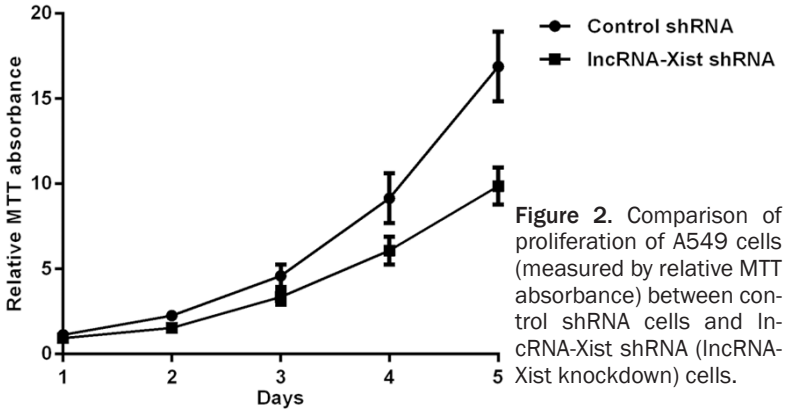


**Figure 2.** Comparison of proliferation of A549 cells (measured by relative MTT absorbance) between control shRNA cells and lncRNA-Xist shRNA (lncRNA-Xist knockdown) cells.

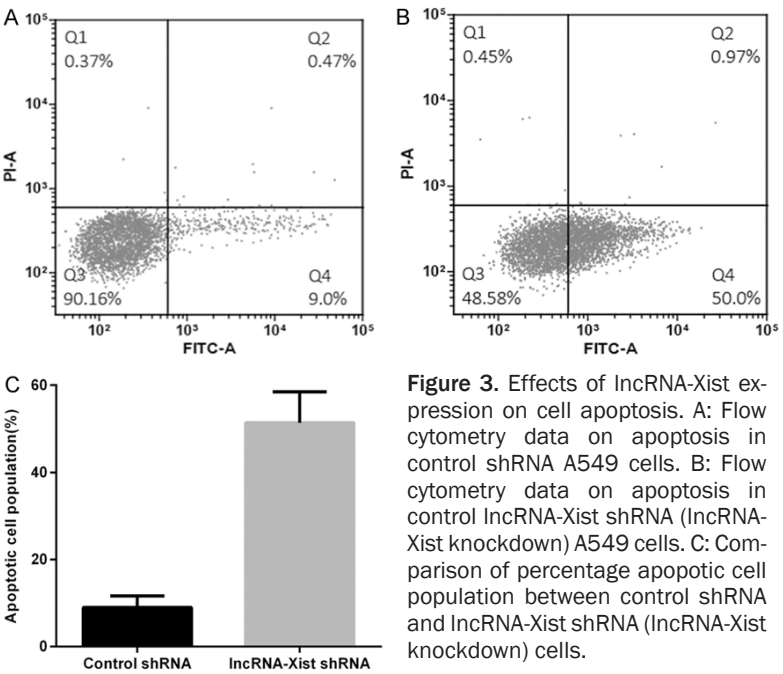


**Figure 3.** Effects of lncRNA-Xist expression on cell apoptosis. A: Flow cytometry data on apoptosis in control shRNA A549 cells. B: Flow cytometry data on apoptosis in control lncRNA-Xist shRNA (lncRNA-Xist knockdown) A549 cells. C: Comparison of percentage apopotic cell population between control shRNA and lncRNA-Xist shRNA (lncRNA-Xist knockdown) cells.

data were represented as mean ± SD. Different groups were analyzed using one way ANOVA. *P*-values <0.05 was considered to be statistically significant.

## Results

*Expression of lncRNA-Xist and miR-103a expression in human lung cancer cells*

RNA was isolated from the surgically resected lung cancer specimens and from the neighboring normal lung tissues (N=60). The expression levels of lncRNA-Xist and miR-103a were measured by real-time PCR as described above. The expression of lnc-RNA XIST was significantly upregulated in human lung cancer cells compared to the normal lung tissues (**Figure 1A**). However, the expression of miR-103a was vice versa which showed a significant down regulated in human lung cancer cells when compared to the normal tissue (**Figure 1B**).

*lncRNA-Xist suppression inhibits the proliferation of A549 cells*

As described earlier shRNA-mediated silencing of lncRNA-Xist was done by infecting the A549 cells by lentivirus infection and then cell proliferation was determined using MTT absorbance. It was found that cell proliferation was significantly suppressed in the human lung cancer cells when compared to that in the normal lung tissue (**Figure 2**).

*lncRNA-Xist suppression induces apoptosis of A549 cells*

shRNA-mediated silencing of lncRNA-Xist (lncRNA XIST knockdown) was performed using lentivirus infection and then flow cytometric analysis (**Figure 3A**) was carried out to measure apoptosis. It was found that the percentage of cells undergoing apoptosis in lncRNA-Xist knockdown cells was significantly higher compared to the normal cells (**Figure 3A-C**).

*lncRNA-Xist suppression mediates the proliferation of human lung cancer A549 cells by regulating the expression of miR-103a*

In the lncRNA-Xist knockdown cells, miR-103a expression, measured by real-time PCR, was found to be significantly higher in comparison to control cells (**Figure 4A**). Furthermore, MTT assay, conducted to determine cell proliferation after ectopic expression of miR-103a mimics (miR103a overexpression) into A549 cells,

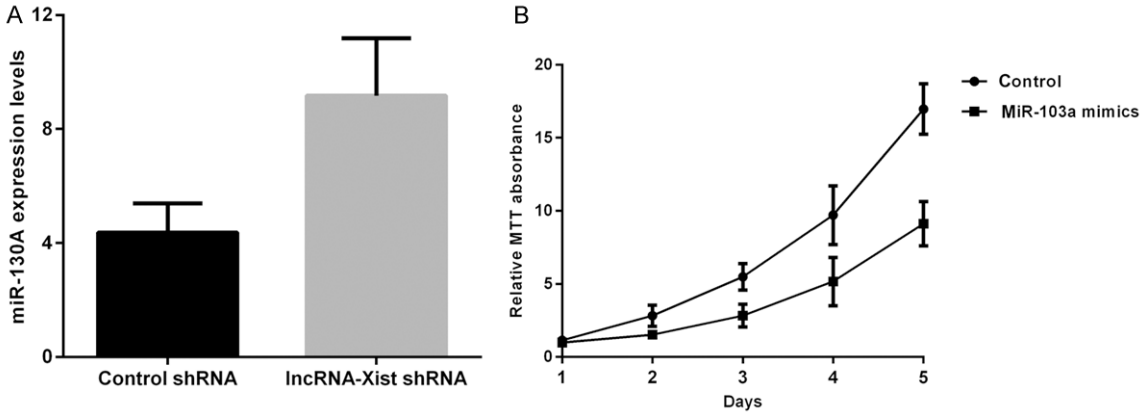


**Figure 4.** lncRNA-Xist regulated lung cancer cell proliferation by mediating miR-103a. A: Comparison of expression of miR-103a between control shRNA and lncRNA-Xist knockdown A549 cells. B: Comparison of proliferation of A549 cells between control shRNA and miR-103a mimics A549 cells.

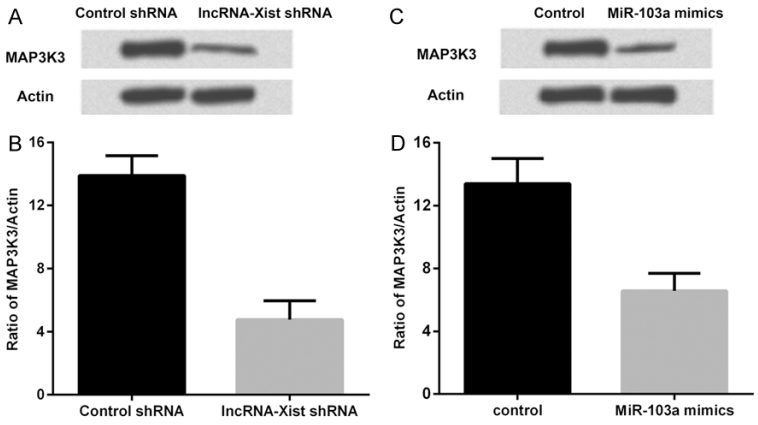


**Figure 5.** lncRNA-Xist expression regulated the MAP3K3 signal pathway in lung cancer cells. A: Comparison of expression of MAP3K3 between control shRNA and in lncRNA-Xist knockdown A 549 cells: Western blot analysis. Actin acted as an internal control. B: Comparison of expression of MAP3K3 between control shRNA and in lncRNA-Xist knockdown A 549 cells. C: Comparison of expression of MAP3K3 between control shRNA and in miR103 a mimics A 549 cells: Western blot analysis. Actin acted as an internal control. D: Comparison of expression of MAP3K3 between control shRNA and in miR103 a mimics A 549 cells.

revealed significant inhibition of A459 cells proliferation compared to the control group (**Figure 4B**).

*lncRNA-Xist suppression and miR-103a overexpression down-regulate the expression of MAP3K3*

Western blot analysis was conducted to examine the expression levels of MAP3K3 after shRNA-mediated silencing of lncRNA-Xist (**Figure 5A**) and ectopic expression of miR-103a mimics (**Figure 5C**) in A549 cells. As shown in the respective figures (**Figure 5A-D**), the expressions of MAP3K3 in both the lncRNA-Xist knockdown cells and overexpressing miR-103a cells were significantly down-regulated when compared to the control group.

## Discussion

The role of lncRNAs in tumorigenesis, invasion, and metastasis has already been established. Different types of lncRNA accounted for different types of cancers. Here, we have found that lncRNA-Xist through modulation of miR-103a and MAP3K3 pathway promotes cellular proliferation in A 549 cell line. Kong R et al, has established that lncRNA PVT1 worsens the prognosis of gastric cancer by promoting the proliferation of cancerous cells through regulating p15 and p16 [28]. Similarly, two studies, which were conducted, independently by two different groups of researchers (Yang MH, et al and JI Q, et al) concluded that lncRNA MALAT1 promotes disease progression in colorectal cancer patients [29, 30]. Similarly, few other studies have supported the progression of cancers by lncRNA (hepatocellular and bladder cancer) [31, 32].

There are number of intracellular programs that are activated following cellular exposure to different external stimuli [21, 33]. The mitogen activated protein kinase (MAPK) is one such intracellular signaling pathway which helps the cells to recognize as well as to respond to number of stimuli. Until now, in mammals five specific members have been identified in the MAPK signaling pathway. These are extracellular signal regulated kinases (ERKs) 1/2, c-Jun amino terminal kinases (JNKs 1, 2, and 3), p38 kinase isoforms (α, β, γ, and δ), ERKS 3, 4 and ERK 5. Of these, ERKS 1/2, JNKs and p38 kinases are the most thoroughly studied members of the MAPK pathway [21].

The MAPK pathway is already known to be associated with a number of cellular functions like cell proliferation, differentiation, survival, and finally death [34]. Any kind of aberrations in the functioning in the MAPK pathway have been associated in the pathogenesis of number of diseases like Alzheimer's disease (AD), Parkinson's disease (PD), amyotropic lateral sclerosis (ALS) and finally various cancers. It has already been established that over activation of the JNK and P38 pathways are associated with neurodegenerative diseases like AD, PD, and ALS whereas the ERK pathway is associated with the different steps in cancer progression such as stimulation of proliferation, migration, and invasion of the malignant cells [34].

Several studies have established that the degree of transcription of lnc RNA is strongly associated with cell differentiation and responsible for the development of metastasis [35, 36]. Studies have also described the striking similarity between fetal tissue development and progression of cancer in terms of overexpression of fetal oncogenes under both the circumstances. Similar to the above findings, it is not surprising that lnc RNAs are also overexpressed in cancer cells in comparison to normal cells as lnc RNA transcription is enhanced in fetal tissue [35].

Huang et al described that lnc RNA DBH-AS1 promotes disease progression (through enhancement of proliferation and survival of cancer cells) through activation of MAPK pathway in hepatocellular carcinoma [35]. Similar to these findings, in our study we have also established that the overexpression of MAPK pathway in A549 cancer cell line is mediated by overexpression of lnc RNA 103a. Another study, conducted by Li R, et al also established the role of lnc RNA mediated MAPK pathway activation in promotion of cancer cell growth [36]. In this study the researchers specifically described that lnc RNA BANCR is responsible for malignant melanoma cell proliferation through activation of MAPK pathway. Similar to our study on NSCLC, Li R, et al found that in malignant melanoma cells silencing of BANCR (mediated by shRNA transfection) suppressed proliferation of the tumor cells and reduced the expression of MAPK pathway [36]. The role of micro RNAs (miRs) as tumor suppressor genes has already been described in different cancers [37-39]. Shin SS, et al, have described the role miR-106a in suppression of progression of bladder cancer [38]. In our study we found that overexpression of miR-103a has led to the suppression of A549 cell line and down-regulation of the MAPK pathway. Similarly, Shin SS, et al, also found that overexpression miR-106a led to tumor suppression in bladder cancer cells through suppression of ERK/MAPK pathway. Again, Wang X, et al found that miR 335 is associated with suppression of bladder cancer through reduced expression of MAPK1 [37].

In this study, we have explored the underlying mechanism through which lncRNA-Xist promotes disease progression in NSCLC. In the human lung cancer cell line A549, the expression of lncRNA-Xist was increased significantly whereas the expression of miR-103a was drastically decreased (**Figure 1A** and **1B**). To deduce the effects of lncRNA-Xist on disease progression in lung cancer cells we measured both proliferation and apoptosis in lncRNA-Xist knockdown A549 cells. It was found that shRNA mediated silencing of lncRNA-Xist in A549 cell line led to favorable outcomes where disease progression decreased significantly (**Figure 2**) while percentage of apoptotic cells significantly increased (**Figure 3B** and **3C**).

Furthermore, in the lncRNA-Xist knockdown A549 cells the expression miR-103a was significantly increased (**Figure 4A**) and overexpression of miR-103a led to suppression of proliferation of A549 cells to a significantly (**Figure 4B**). These results suggested proliferation of lncRNA-Xist knockdown A459 cells was suppressed by overexpression of miR-103a.

Finally, it was found that lnc RNAXIST knockdown and miR-103a overexpression led to decreased expression of MAP3K3 in A549 cells (**Figure 5A-D**, respectively).

The role of lncRNA as an early diagnostic tool in different types of cancer has already been established. The expression of lncRNA not only aids in diagnosing but also acts as a prognostic factor in varied types of cancer [5, 8].

In conclusion, our study demonstrated that overexpression of lnc RNAXIST in the A549 cells promotes disease progression by up-regulation of MAP3K3 pathway and at the same time, is responsible for the decreased expression of miR-103a which independently suppresses the proliferation and down-regulation of MAP3K3 pathway in cancerous A549 cells.

Understanding the underlying mechanism of lncRNA in NSCLC might provide the basis for development of novel drug targets in near future.

## Disclosure of conflict of interest

None.

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